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Cardiovascular Pharmacology of 3-*n*-butylphthalide in Spontaneously Hypertensive Rats

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The hypotensive and vasorelaxant effects of 3-*n*-butylphthalide (BuPh) and its possible mechanisms of action were investigated in spontaneously hypertensive rats (SHR) for the first time. A 13-day intraperitoneal infusion of BuPh at doses of 2.0 and 4.0 mg/day produced a transient hypotensive effect while a dose of 0.5 mg/day showed a significant hypotensive effect only on day 12. BuPh at 0.5 mg/day had no effect on the plasma and tissue angiotensin converting enzyme (ACE) activities, or on the tissue lipid peroxidation index. BuPh relaxed endothelium-intact and denuded aortic rings precontracted with phenylephrine and KCl. N^G-nitro-L-arginine methyl ester, an inhibitor of nitric oxide synthase, did not attenuate the vasorelaxant activity of BuPh. The cumulative concentration response curves of phenylephrine and Ca^{2+} (in $CaCl_2$-free, high KCl medium) were non-competitively inhibited by BuPh. However, BuPh did not interfere with the caffeine-induced release of intracellular Ca^{2+}. It appears that the vasorelaxant effect of BuPh could be attributed to the blockade of Ca^{2+} entry, possibly through voltage- and receptor-operated Ca^{2+} channels, thereby lowering the systolic blood pressure of SHR. © 1997 John Wiley & Sons, Ltd.

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INTRODUCTION

Celery (*Apium graveolens*) is a vegetable that is commonly eaten in the local diet. It has long been recommended in traditional Chinese medicine as a natural food cure for the cleansing of blood and treatment of hypertension (Lu, 1986). In a preliminary study, a decoction of celery was shown to decrease the blood pressure of renovascular occlusive hypertensive dogs (Baramee and Lohsiriwat, 1987). Intraperitoneal (i.p.) administration of aqueous celery extract via osmotic pumps to spontaneously hypertensive rats (SHR) showed a delayed but significant hypotensive effect (Tan and Hsu, 1991).

BuPh (Fig. 1a), a chemical unique in celery, is a small lipophilic molecule with a molecular weight of 190. Together with other phthalides, it contributes to the characteristic flavour of celery (Uhlig *et al.*, 1987). Le and Elliott (1991) reported that BuPh decreased the systolic blood pressure (SBP) of normotensive Sprague Dawley rats significantly after daily i.p. injection for a fortnight. They also showed that BuPh caused relaxation of canine femoral arterial rings precontracted with KCl and noradrenaline but did not investigate the mechanism(s) of action (Le and Elliott, 1992).

The consistent hypotensive activity exhibited by BuPh prompted us to carry out further in depth investigations on its mechanisms of action. BuPh can serve as a potential lead for the development of a new antihypertensive agent. No work has been done to study further the hypotensive effect of BuPh and its possible mechanism(s) of action in a genetically hypertensive animal model such as the SHR. This is important as one cannot assume that BuPh will exert the same hypotensive effect in SHR as in the normotensive SD rats. The SHR is a strain of genetically hypertensive rat which was developed by Okamoto and Aoki (1963) and has been widely used as an animal model for human essential hypertension as it shares many pathophysiological characteristics with human essential hypertension (Frohlich, 1986). In this study, we investigated the effects of a 13-day i.p. infusion of BuPh on the SBP, and related biochemical parameters in conscious SHR as well as the vasorelaxant effect of BuPh on SHR thoracic aortic rings in order to determine the mechanisms of action of BuPh.

MATERIALS AND METHODS

Chemicals. BuPh was synthesized (in excess of 97% purity) and supplied by Professor W. J. Elliott of Rush University,

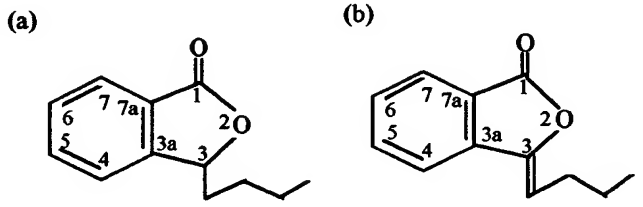

Figure 1. Chemical structures of (a) 3-*n*-butylphthalide and (b) 3-butylidenephthalide.

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Illinois, USA. Phenylephrine, caffeine, dimethyl sulphoxide (DMSO), hippuryl-L-histidyl-L-leucine and thiobarbituric acid were obtained from Sigma Chemical Co. (St. Louis, MO, USA) while N^G-nitro-L-arginine methyl ester (L-NAME) was purchased from RBI Research Biochemicals International (MA, USA). All other chemicals used were of the best analytical grade.

***In vivo* experiments.** Male SHR aged about 15 weeks were imported from the Animal Resource Center, Perth, Western Australia. The rats were housed in groups of four to five in perspex cages with free access to food and water and allowed to acclimatize for at least 1 week before the start of experiments.

Four doses of BuPh (0.5, 1.0, 2.0 and 4.0 mg/day) were infused i.p. into SHR rats (mean body weight of 300 g) for 13 days via polyethylene (PE 60) cannulas connected to Alzet oxmotic pumps (ALZA Corporation, CA, USA). Control SHR rats were infused with the vehicle, ethanol–propylene glycol (7:3 v/v). The systolic blood pressure of each rat was measured by the indirect tail cuff method (Bunag, 1973) using a photoelectric sensor connected to a pulse amplifier (IITC Inc., CA, USA) on every alternate day until day 12. Body weight, food and water intake, as well as urine output of each rat were monitored daily.

On day 13, the rats treated with 0.5 mg of BuPh were killed in order to obtain the plasma, aortas, lungs and kidneys. The lungs and kidneys were homogenized with a glass Potter-Elvejhem homogenizer in 10 volumes of 0.1 M potassium phosphate buffer, pH 8.3 containing 0.3 M NaCl. The homogenate was then filtered through a layer of cotton to remove connective tissue (Sim and Chua, 1987). The activities of angiotensin converting enzyme (ACE; EC 3.4.15.1) in these samples were measured according to Cushman and Cheung (1971).

The aorta and a portion of the kidney sample from each rat were homogenized in ice-cold 1.15% KCl using a polytron homogenizer. The lipid peroxidation index of the samples was then determined by the thiobarbituric acid method of Uchiyama and Mihara (1978) as described by Ohtawa *et al.* (1983).

Aortic rings. Thoracic aortas were obtained from SHR aged 18–20 weeks old. The aorta (about 3 cm long) was immediately excised after decapitation of the rat. While immersed in aerated Krebs solution, the aorta was trimmed free of adhering fat and connective tissues before cutting into rings of about 2 mm each. Each ring was mounted on two fine stainless steel hooks in a 10 mL organ bath containing Krebs solution (composition, mM: NaCl, 118; KCl, 5; $NaHCO_3$, 25; glucose, 10; $CaCl_2$, 2.5; $MgSO_4.7H_2O$, 1.2; KH_2PO4, 1.2; EDTA, 0.025) aerated with 95% O_2 and 5% CO_2 and maintained at 37°C. One hook was connected via a silk thread to an isometric transducer (Ugo Basille, Italy) coupled to a polygraph (Grass Co., Mass, USA) while the other hook was attached to the base of the gas inlet steel tubing. All the aortic rings were subjected to a resting tension of 1 g and equilibrated for at least 60 min prior to any experiment. During this period, Krebs solution was changed every 20 min. In all experiments the vehicle, 0.1% DMSO (final concentration), was added to a separate set of aortic rings which served as controls.

Effects of BuPh on phenylephrine and high KCl-induced contraction. In these experiments, both endothelium-intact and denuded aortic rings were used to determine whether the endothelium was involved in the vascular effect of BuPh. The denuded aortic rings were prepared by inserting the tip of a small pair of forceps into the luminal surface of the ring and rolling it back and forth for about 20 s on filter paper soaked with Krebs solution. The aortic rings were considered to have intact functional endothelium when they showed a relaxation of 70% or more in response to 10^{-6} M acetylcholine after being contracted with 10^{-7} M phenylephrine.

Each aortic ring was contracted with either 10^{-7} M phenylephrine or 80 mM KCl. When the ring was at the tonic contraction phase for about 10 min, BuPh was added cumulatively (5×10^{-5}–4×10^{-4} M). Each ring was allowed to reach a new stable tension before addition of subsequent concentrations of BuPh. The relaxation produced by cumulative doses of BuPh was expressed as a percentage of inhibition of the maximum tonic contraction induced by 10^{-7} M phenylephrine or 80 mM KCl.

Effect of N^G-nitro-L-arginine methyl ester (L-NAME) on the activity of BuPh on phenylephrine-induced contraction. Each ring was incubated with the specific nitric oxide synthase inhibitor, L-NAME (25 μM), for about 20 min before being contracted by 10^{-7} M phenylephrine. After the tonic response was stable, cumulative doses of BuPh (5×10^{-5}–4×10^{-4} M) were added. The control rings were similarly tested but without preincubation in L-NAME. The relaxation produced by cumulative doses of BuPh was calculated as mentioned above.

Effect of BuPh on cumulative concentration-response curves to phenylephrine and $CaCl_2$. Cumulative concentrations of phenylephrine (10^{-9}–10^{-5} M) were added to the bath to obtain the basal concentration-response curves. After a stabilization period of 60 min with three washings in between, the aortic rings were exposed for about 20 min to 0.1% DMSO or three different doses of BuPh (2.5, 25 and 150 μM); each ring was exposed to only one concentration of BuPh. The second cumulative concentration-response curves for phenylephrine were then obtained.

In another experiment, the aortic rings were exposed for 60 min to $CaCl_2$-free, high-KCl solution (prepared by equimolar replacement of NaCl with 50 mM KCl) with three washings in between. Cumulative concentration-response curves to Ca^{2+} (0.03–3 mM) were then obtained in the absence and presence of different doses of BuPh (2.5, 25, 100 μM) and 0.05 μM verapamil. The contractile responses obtained after treatment with 0.1% DMSO or BuPh were expressed as percentages of the basal response induced by phenylephrine and $CaCl_2$.

Effect of BuPh on caffeine-induced contraction in $CaCl_2$-free Krebs solution. After the initial equilibration period, the aortic rings were bathed in $CaCl_2$-free Krebs solution for 15 min. A rapid transient contraction elicited by 10 mM caffeine was considered as the initial basal contractile response. The caffeine was washed out with $CaCl_2$-free Krebs solution followed by a 20 min resting period in normal Krebs solution. These rings were then pretreated with 0.1% DMSO or 150 μM BuPh for about 20 min in $CaCl_2$-free Krebs solution before adding caffeine. The contractile responses obtained after treatment were expressed as percentages of the basal response induced by caffeine.

Data and statistical analysis. All data were expressed as mean±SEM. The percentage changes in SBP of SHR over the 12 days were analysed by 2-way ANOVA followed by Duncan's Multiple Range Test using the Statistical Analysis System (SAS) Package. The data on ACE activities, lipid peroxidation index and daily urine output were analysed by Student's *t*-test. In the organ bath studies, the ED_{50} values of BuPh were analysed by Student's *t*-test. The data on the concentration-response curves of phenylephrine and $CaCl_2$ were analysed by 1 way ANOVA followed by Dunnett's test. A difference at $p<0.05$ was considered to be statistically significant.

RESULTS

In vivo experiment

The SBP of the animals ranged from 175 to 219 mmHg at the beginning of the experiment. On day 2, rats treated with 2.0 and 4.0 mg BuPh/day but not those treated with 0.5 and 1.0 mg BuPh/day showed a significant decrease in SBP relative to the controls (Fig. 2). From day 4 onwards, the SBP of all the treatment groups, except the 0.5 mg/day group, returned to pre-treatment or even higher levels. Interestingly, only the 0.5 mg/day dose significantly lowered the SBP at the end of the experiment.

The ACE activities of plasma, lung and kidney of the 0.5 mg BuPh treated group were 1.55±0.14 ($n=6$), 8.78±0.40 ($n=6$) and 0.35±0.09 ($n=4$) μmole/min/g wet weight respectively, while those of the control group were 1.38±0.18 ($n=6$), 9.22±0.82 ($n=6$) and 0.21±0.02 ($n=3$) respectively. There were no significant differences between the two groups.

In the treated SHR, the thiobarbituric acid-reacting substances (TBARs) estimated in the aorta and kidney were 0.15±0.05 ($n=6$) and 1.97±0.09 ($n=6$) μmole/g wet weight respectively, compared with 0.11±0.01 ($n=6$) and 2.03±0.08 ($n=6$) μmole/g wet weight respectively in the control SHR. No significant differences were observed between the two groups.

The mean daily urine output of the treated SHR was not significantly different from that of the controls throughout the experimental period, being 1.5±0.2 and 1.8±0.2 mL/100 g body weight respectively.

In vitro experiments

At concentrations between 5×10^{-5} and 4×10^{-4} M of BuPh, the basal tone of aortic rings in Krebs solution was not affected. DMSO at a final concentration of 0.1% did not affect the tension of aortic rings preconcontracted with 10^{-7} M phenylephrine and 80 mM KCl.

BuPh produced a concentration-dependent relaxation in aortic rings precontracted with 10^{-7} M phenylephrine (Fig. 3a). The relaxation effect of BuPh in endothelium-intact rings was not significantly different from that in the endothelium-denuded rings (ED_{50} values being $7.3\pm0.8\times10^{-5}$ M and $9.7\pm1.0\times10^{-5}$ M, respectively). At the maximum dose of 4×10^{-4} M, BuPh relaxed both the endothelium-intact and denuded aortic rings almost completely (99%).

Figure 3b shows the preincubation of the aortic rings with L-NAME had no effect on the BuPh-induced relaxation. The ED_{50} values of BuPh in control rings ($7.3\pm0.8\times10^{-5}$ M) and rings preincubated in L-NAME ($5.9\pm0.7\times10^{-5}$ M) were not significantly different. The phenylephrine-induced contraction in L-NAME pretreated aorta was completely abolished when the maximum concentration of BuPh (4×10^{-4} M) was used.

Pretreatment of aortic rings with three different concentrations of BuPh (2.5, 25 and 150 μM) depressed the maximum contractile response of phenylephrine-induced concentration-response curves in a non-competitive manner (Fig. 4).

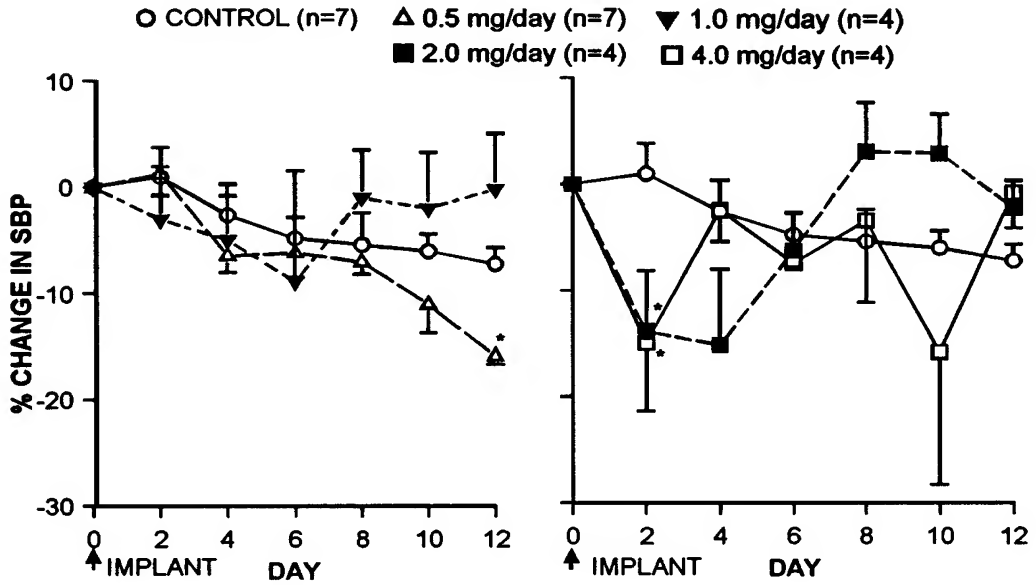

Figure 2. Changes in SBP of spontaneously hypertensive rats (SHR) over a 12-day period of i.p. infusion of different doses of BuPh and vehicle (ethanol:propylene-glycol; 7:3) by osmotic pumps. Vehicle-treated:○ ($n=7$). BuPh-treated: 0.5 mg/day △ ($n=7$); 1.0 mg/day ▼ ($n=4$); 2.0 mg/day ■ ($n=4$); 4.0 mg/day □ ($n=4$). Each point represents the mean of 4 or 7 rats, while the bar indicates SEM. *$p<0.05$: Significantly different from the vehicle-treated group (Duncan's Multiple Range test).

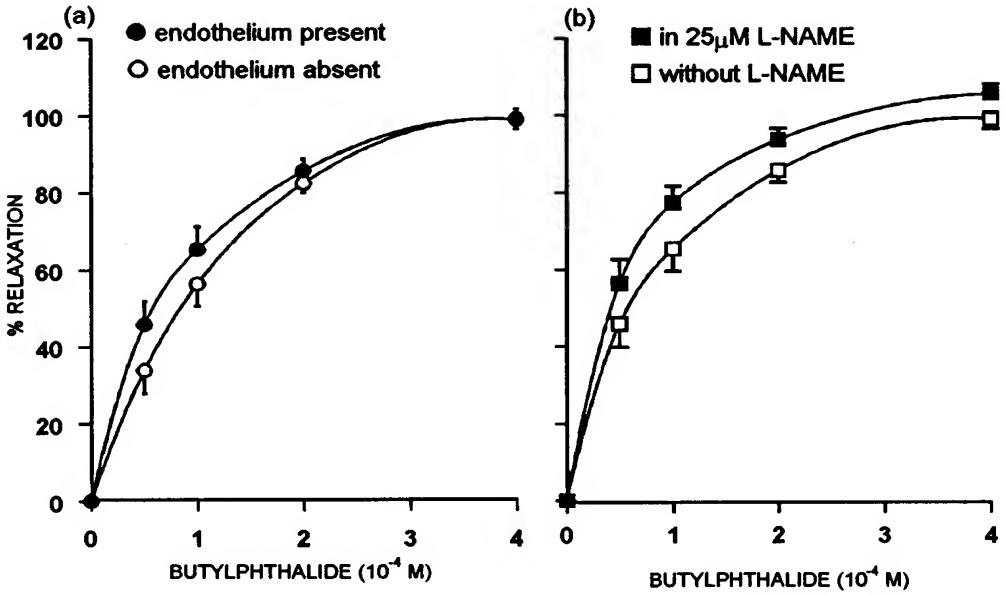

Figure 3. Vasorelaxation induced by cumulative concentrations of BuPh in SHR aortic rings precontracted with 10^{-7} M phenylephrine. (a) in the presence (●) or absence (○) of endothelium. (b) with (■) or without (□) a prior 20 min incubation in 25 μM N^G-nitro-L-arginine methyl ester. Values are expressed as the percentages of inhibition of the maximal tonic contractile responses induced by 10^{-7} M phenylephrine. Each point represents the mean of 9–11 experiments while the bars indicate the SEM.

Figure 5 shows that the sustained contractile response to 80 mM KCl was inhibited by BuPh in a dose dependent manner even in the absence of endothelium. The ED_{50} values of BuPh were not significantly affected by the removal of endothelium, being $11.8\pm0.8\times10^{-5}$ M in endothelium-intact aorta compared with $12.2\pm1.0\times10^{-5}$ M in the denuded aorta. BuPh at 4×10^{-4} M relaxed the denuded aorta beyond the resting tension (105%) and produced a 90% relaxation of the intact aorta.

Following preincubation in a $CaCl_2$-free high-KCl depolarizing Krebs solution for 20 min, BuPh (2.5, 25 and 100 μM) inhibited Ca^{2+}-induced contraction in a concentration-dependent manner and caused a downward shift of the Ca^{2+} concentration-response curves (Fig. 6). 0.05 μM verapamil also inhibited the Ca^{2+} concentration-response curve and its effect appeared to be comparable to that of 100 μM BuPh (Fig. 6).

Pretreatment of aortic rings with either 0.1% DMSO or

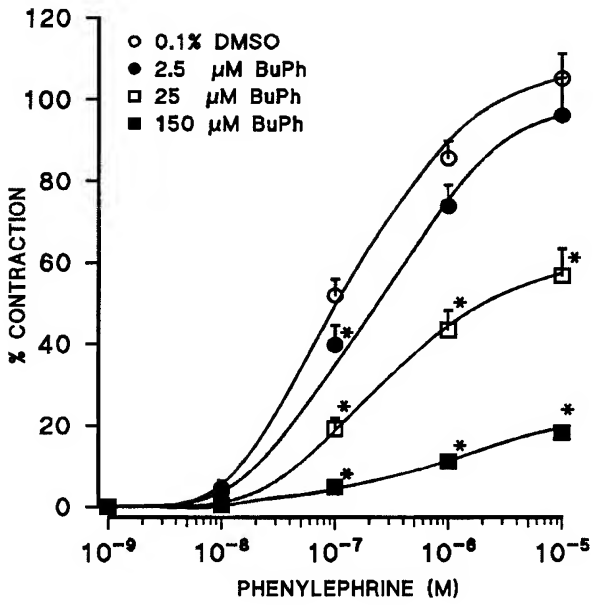

Figure 4. Concentration-response curves to phenylephrine in SHR aortic rings in the presence of 0.1% DMSO (○) or BuPh at 2.5 μM (●), 25 μM (□) and 150 μM(■). Values are expressed as the percentages of basal contractile responses induced by cumulative doses of phenylephrine before aortic rings were treated with BuPh. Each point represents the mean of 8–10 experiments while the bars indicate the SEM $*p<0.05$: Significantly different from the control (Dunnett's test).

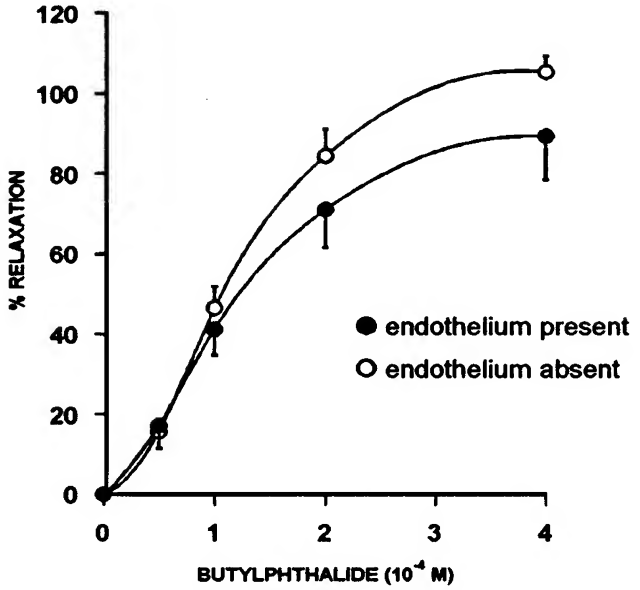

Figure 5. Vasorelaxation induced by cumulative concentrations of BuPh in SHR aortic rings precontracted with 80 mM KCl in the presence (●) or absence (○) of endothelium. Values are expressed as the percentages of inhibition of the maximal tonic contractile responses induced by 80 mM KCl. Each point represents the mean of 6–7 experiments while the bars indicate the SEM.

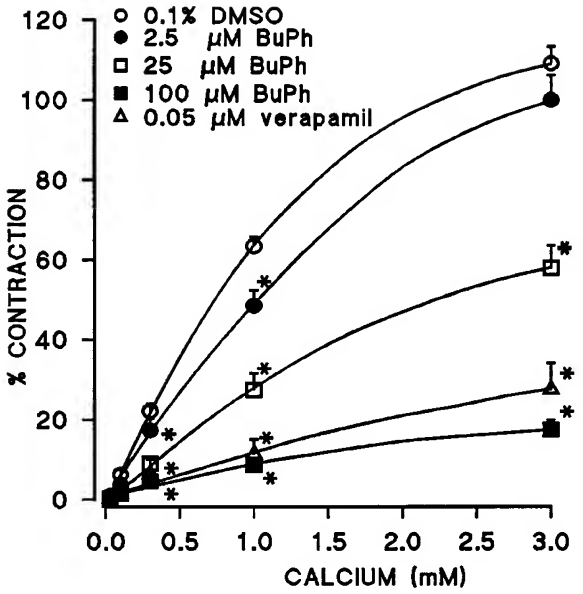

Figure 6. Concentration-response curves to Ca^{2+} in SHR aortic rings in $CaCl_2$-free, high KCl depolarizing Krebs solution in the presence of 0.1% DMSO (○) or BuPh at 2.5 μM (●), 25 μM (□) and 100 μM (■) and verapamil 0.05 μM (△). Values are expressed as the percentages of basal contractile responses induced by increasing doses of Ca^{2+} before aortic rings were treated with BuPh. Each point represents the mean of 7–11 experiments while the bars indicate the SEM. *$p<0.05$: Significantly different from the control (Dunnett's test).

150 μM BuPh did not affect caffeine-induced contraction. The contractile response to 10 mM caffeine was 117.8%±13.9% in DMSO pretreated aorta and 126.4%±12.1% in BuPh pretreated aorta, relative to the basal caffeine-induced contraction.

DISCUSSION

The data on conscious SHR showed that at higher doses of 2.0 and 4.0 mg/day, the hypotensive effect of BuPh was not sustained throughout the 13-day period of i.p. infusion. However, 0.5 mg/day BuPh decreased the SBP of SHR significantly at the end of the experiment, indicating a slow onset of action. The question arises as to whether the pumps were functioning well or the decrease in SBP on day 2 was an artifact. We have examined the osmotic pumps by cutting them open and found that the pumps were functioning properly as the inner reservoir had collapsed. The decrease in SBP on day 2 was not an artifact (Fig. 2) as the SBP of the control rats remained very much unchanged while those treated with 2 and 4 mg BuPh/day groups showed a clear-cut decrease. Also, the SBP of the two groups of rats treated with lower doses of BuPh (0.5 and 1.0 mg/day) were very similar to pre-experimental readings. At present we do not know the reasons for the development of tolerance to the hypotensive effect of higher doses of BuPh as it involves complex biochemical and physiological mechanisms. The desenitization observed seemed to be dose and time dependent, which also occurred in other drugs, as demonstrated by Thadani *et al.* (1982). The study showed that nitrate administration did not have a long term effect in prolonging the exercise time of angina patients. Thadani *et al.* (1987) also showed that sustained release formula of 1–5-mononitrate did not prevent the occurrence of tolerance. In another study, tolerance to the inhibitory actions of salbutamol (a β-adrenoceptor agonist) and diltiazem (a calcium antagonist) on uterine contractions in ovariectomized, post-partum rats occurred after a 20 h intravenous infusion (Abel and Hollingsworth, 1986). It is possible that increased production of angiotensin II and a more sensitive baroreflex response of the SHR could be responsible for the rebound of SBP from day 4 onwards. In contrast to our study, Le and Elliott (1992) reported the hypotensive effect of BuPh when administered daily to SD rats by i.p. injection for 4 weeks. The differences in observation may be due to different strain and the mode of administration used. Also, this may indicate that bolus administration of BuPh could be more effective than continuous infusion in the sustained lowering of SBP.

From the data on ACE activities, it appears that BuPh did not exert its hypotensive effect by inhibiting circulating or tissue ACE activities. The normal daily urinary output also suggests that BuPh did not have a diuretic effect.

Lipid peroxidation is the process of oxidative deterioration of polyunsaturated fatty acids leading to the formation of free radicals (Gutteridge and Halliwell, 1990). These free radicals are known to cause cellular injuries and it has been reported that there was an increased level of lipid peroxides in patients with essential hypertension (Prabha *et al.*, 1990). Lipid peroxidation in SHR tissues was, however, not affected by the administration of BuPh, indicating that BuPh, unlike tannic acid (Yugarani *et al.*, 1993), did not have beneficial effects on free radical-mediated cellular injury in the SHR.

The vascular endothelium plays a crucial role in maintaining the tone of blood vessels by releasing endothelium-derived relaxation factor (EDRF), prostacyclins, hyperpolarizing factor and vasoconstrictor factors, such as endothelin (Vanhouette *et al.*, 1986; Kuriyama *et al.*, 1995). It is now established that nitric oxide is the EDRF which is endogenously synthesized from L-arginine by nitric oxide synthase (Moncada *et al.*, 1991). BuPh caused vasorelaxation in SHR aortic rings precontracted with 10^{-7} M phenylephrine and 80 mM KCl in a concentration-dependent manner. Moreover, this relaxant effect was shown to be independent of endothelium. Also, the observation that pretreatment of aortic rings with the specific competitive inhibitor of nitric oxide synthase, L-NAME, did not attenuate the vasorelaxant effect of BuPh suggests that BuPh relaxed phenylephrine-contracted aortic rings by acting directly on the smooth muscles without the mediation of nitric oxide.

Very recently, Villalobos-Molina and Ibarra (1996) reported that the α_{1A} or α_{1D} receptor subtypes were responsible for the α_1-adrenoceptor-mediated contraction in the arterial rings of the SHR. Phenylephrine activates the α_1-adrenoceptor, leading to the rapid release of intracellular Ca^{2+} from endoplasmic reticulum by inositol-1,4,5-triphosphate (Hashimoto *et al.*, 1986; Takata and Kato, 1996). This is followed by the influx of extracellular Ca^{2+} through receptor-operated Ca^{2+} channels (Bolton, 1979; Godfrained *et al.*, 1986) giving rise to tonic contraction. Cumulative doses of BuPh caused relaxation of phenylephrine-induced contraction at the tonic phase. We also found that BuPh suppressed the maximal contractile response to phenylephrine in a concentration-dependent manner. The phenylephrine concentration-response curves were also shifted downwards in a manner that was characteristic of non-competitive antagonism. This indicates that that BuPh could be affecting the receptor-operated Ca^{2+} channels.

The contractile responses induced by high KCl (80 mM) or by graded increase of Ca^{2+} in KCl-depolarized vascular smooth muscles are due to the influx of extracellular Ca^{2+} through the L-type voltage-operated Ca^{2+} channels that are sensitive to compounds like verapamil and nifedipine (Karaki and Weiss, 1988; Godfraind *et al.*, 1989). We showed that BuPh not only inhibited the tonic contraction of aortic rings elicited by 80 mM KCl but also suppressed the Ca^{2+}-dependent contraction of the aortic rings in $CaCl_2$-free, high KCl depolarizing medium in a concentration-dependent manner. These findings suggest that BuPh blocked extracellular Ca^{2+} influx through the voltage-operated channels as well. Other compounds derived from natural products such as apigenin and quercitin have also been shown to exhibit such Ca^{2+} blockade property (Ko *et al.*, 1991; Morales and Lozoya, 1994).

Apart from tonic contraction due to extracellular Ca^{2+} influx, vascular smooth muscles can also undergo rapid transient contraction by the mobilization of intracellular Ca^{2+} from the sarcoplasmic reticulum in response to agents like caffeine (Karaki and Weiss, 1988). We observed that BuPh did not interfere with the release of intracellular Ca^{2+} from the caffeine-sensitive Ca^{2+} pool.

Taken together, the *in vivo* and *in vitro* data indicate that BuPh could be blocking the influx of extracellular calcium through the voltage- as well as receptor-operated (mediated by non-competitive antagonism of α_1 adrenoceptor) Ca^{2+} channels of SHR aortic smooth muscle cells to cause vasorelaxation. It is likely that this calcium blockade property of BuPh may be responsible for the hypotensive effect observed in the conscious SHR rats.

Besides BuPh, 3-butylidenephthalide, another compound in celery, has recently been reported to decrease the blood pressure of renal hypertensive rats (Ko *et al.*, 1994). It has a chemical structure similar to BuPh, except for a double bond instead of a single bond at position 3 connecting to the butyl side chain (Fig. 1a and b). As both these closely related compounds have hypotensive activity, it is our future plan to synthesize and investigate other analogues for hypotensive property in order to define the structure–activity relationship of these phthalides. Our interesting finding warrants further investigation of BuPh as a lead compound for the development of other more potent analogues.

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